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Toxoplasma gondii Antigen-Pulsed-Dendritic Cell-Derived Exosomes Induce a Protective Immune Response against *T. gondii* Infection

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It was previously demonstrated that immunizing mice with spleen dendritic cells (DCs) that had been pulsed ex vivo with *Toxoplasma gondii* antigens triggers a systemic Th1-biased specific immune response and induces protection against infection. *T. gondii* can cause severe sequelae in the fetuses of mothers who acquire the infection during pregnancy, as well as life-threatening neuropathy in immunocompromised patients, in particular those with AIDS. Here, we investigate the efficacy of a novel cell-free vaccine composed of DC exosomes, which are secreted antigen-presenting vesicles that express functional major histocompatibility complex class I and II and T-cell-costimulatory molecules. They have already been shown to induce potent antitumor immune responses. We investigated the potential of DC2.4 cell line-derived exosomes to induce protective immunity against toxoplasmosis. Our data show that most adoptively transferred *T. gondii*-pulsed DC-derived exosomes were transferred to the spleen, elicited a strong systemic Th1-modulated *Toxoplasma*-specific immune response in vivo, and conferred good protection against infection. These findings support the possibility that DC-derived exosomes can be used for *T. gondii* immunoprophylaxis and for immunoprophylaxis against many other pathogens.

Toxoplasma gondii is an obligate intracellular parasite that infects a wide range of hosts, including humans and domesticated animals throughout the world. It initially invades the intestinal epithelium, and if the infectious form is acquired during pregnancy, it can lead to insidious and serious fetal damage and may occasionally cause fatal diseases. In patients suffering from AIDS or other immunocompromising conditions, the reactivation of chronic toxoplasmosis results in severe cell destruction, often leading to severe morbidity and mortality (21).

In veterinary medicine, *T. gondii* infection has economic importance since it can cause abortion in and neonatal loss of sheep and goats. Thus, the development of an effective vaccine against *T. gondii* would be of great value to both human and veterinary medicine. The rationale for developing an effective vaccine is that primary infection leads to the acquisition of permanent protective immunity against reinfection.

Following a primary infection in an immunocompetent individual, the immune response of the host usually limits the replication of tachyzoites by means of a systemic Th1 response, resulting in the formation of the bradyzoite form characteristic of the chronic stage of the infection. *T. gondii* infection generates early cellular immunity, which is mediated by dendritic cells (DCs) and permits the development of the antigen-specific response by directing a switch from helper T cells to Th1 lymphocytes at the systemic level. Both $CD4^+$- and $CD8^+$-T-cell subsets mediate protective immunity (2, 19), and this protective immunity depends mainly on the ability of T cells to produce gamma interferon (IFN-γ) (30).

DCs are professional antigen-presenting cells (APCs) with the essential properties required for the effective induction of an immune response (38). DCs are therefore thought to play a key role in immunity. Interleukin 12 (IL-12) is secreted by DCs and plays a crucial role in triggering the protective immune response by promoting IFN-γ production. Several studies have demonstrated the efficacy of DC-based antitumor vaccines (5, 14, 22, 25, 26) and anti-infectious vaccines in viral (12, 27, 28), bacterial (13, 15, 16, 17, 29), and parasitic (6, 36) models. In our *T. gondii* model, we demonstrate that adoptively transferred spleen or mesenteric lymph node DCs pulsed in vitro with *T. gondii* antigens induce a *Toxoplasma*-specific immune response in vivo and confer good protection against the acute and chronic stages of the infection (4).

The aim of the present study was to optimize the immune responses induced by DCs. In addition to direct interaction and the secretion of cytokines, DCs are also able to trigger T-cell responses by producing exosomes (20, 32, 39). Multivesicular major histocompatibility complex (MHC) class II molecule-rich compartments of DCs can fuse with the plasma membrane, resulting in the release of antigen-presenting vesicles, or exosomes, into the extracellular environment (20). These exocytosed vesicles express MHC molecules of classes I and II and are able specifically to stimulate $CD4^+$-T-cell proliferation via the MHC molecule-peptide complex presented by exosomes or via the capture of DC-derived exosomes by other APCs (20, 32). The identification of major exosomal components (MAC-1, milk fat globule [MFG]-E8/lactadherin, and CD9) that display affinity with ligands on other cell

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membranes (intracellular adhesion molecule 1 [ICAM-1] and ICAM-2, $\alpha_v\beta3$ and $\alpha_v\beta5$, and epidermal growth factor [EGF]-like receptor, respectively) confirms this hypothesis (33).

Exosomes have been shown to induce potent antitumor immune responses in vivo. Indeed, a single injection of DC-derived exosomes sensitized with tumor peptides induced the eradication of established mouse tumors (39). These findings strongly suggest the potential of DC-derived exosomes in cancer immunotherapy. A clinical trial in which patients with metastatic melanoma or inoperable lung cancer are vaccinated with autologous DC-derived exosomes pulsed with Mage-3 peptides is under way (32).

Here we demonstrate that most of the *T. gondii* antigen-pulsed DC-derived exosomes are transferred to the intestine before homing to the spleen and that they prime an antigen-specific cellular and humoral immune response that provides good protection against both acute and chronic toxoplasmosis. This is the first demonstration that exosomes can induce a protective immune response against pathogens. A cell-free vaccine of this type is an attractive potential tool for a human vaccination strategy.

MATERIALS AND METHODS

Animals. Female C57BL/6 (*H-2^b*) mice aged 8 to 10 weeks (Janvier) were used in all experiments. The C57BL/6 strain was chosen for the analysis of both the chronic and acute stages of infection. In this model, mice orally infected with 30 cysts of the 76K strain of *T. gondii* do not limit the replication of tachyzoites, and they die during the acute stage of infection. However, C57BL/6 mice orally infected with 10 cysts survive and long-term protection can be studied by counting their brain cysts 1 month after infection.

Parasites. Tachyzoites of the RH strain of *T. gondii* were harvested from the peritoneal fluid of Swiss OF1 mice that had been intraperitoneally infected with 2×10^6 tachyzoites 3 to 4 days earlier. Cysts of the 76K strain of *T. gondii* were obtained from the brains of Swiss OF1 mice that had been orally infected with 80 cysts 1 month earlier.

Preparation of the *Toxoplasma* sonicate. The *T. gondii* RH tachyzoites were washed, sonicated, and centrifuged as previously described (23). The concentration of the sample was determined by using a protein assay reagent kit (Bio-Rad, Marnes La Coquette, France) with bovine serum albumin as the standard. The aliquots of *T. gondii* tachyzoite sonicate, called *Toxoplasma* antigens (TAgs), were stored at −20°C.

Cell surface markers of TAg-pulsed or unpulsed DC2.4 cells. The DC2.4 cell line (*H-2^b*), immortalized by using a protocol described by Shen et al. (24), was kindly provided by K. L. Rock (Dana-Farber Cancer Institute, Boston, Mass.).

In order to analyze the effect of TAg activation on the expression of DC2.4 cell surface markers, 2×10^6 DC2.4 cells were resuspended in 15 ml of Iscove's modified Dulbecco medium (GIBCO/BRL) supplemented with L-glutamine (2 mM) (GIBCO/BRL), 1% penicillin-streptomycin (GIBCO/BRL), and 5% heat-inactivated fetal calf serum (FCS) (GIBCO/BRL); filtered with a 0.22-μm-pore-size filter; and then cultured in 75-cm^3 flasks (Corning). These DCs were pulsed with 50 μg of TAg/ml for 18 h. The expression of the surface molecules was quantified by flow cytometry using the following monoclonal antibodies (MAbs): 14.4.4 (mouse immunoglobulin G2a [IgG2a] anti-I-E^{k,d}), 53-2-1 (IgG2a anti-mouse CD90.2), 16-10A1 (rat IgG2a anti-mouse CD80), GL1 (hamster anti-mouse CD86), Ly-2 (IgG2a anti-mouse CD8α), and 3/23 (Rat IgG2a anti-mouse CD40) fluorescein isothiocyanate-conjugated MAbs (all from Pharmingen) and the phycoerythrin-conjugated MAb MI/70 (rat anti-mouse MAC-1). Before undergoing specific staining, aliquots of 10^6 cells were incubated for 10 min with 2.4G2 antibody (rat anti-mouse Fc receptor MAb; Pharmingen) to prevent the antibody from binding to the Fc receptors during the flow cytometry. The cells were washed twice with phosphate-buffered saline (PBS)–1% FCS (vol/vol) and incubated with the specific MAb or with the corresponding isotype control (1 μg/10^6 cells/100 μl, diluted in PBS–1% FCS) for 30 min at 4°C. The cells were washed twice with PBS–1% FCS (vol/vol) and suspended in 500 μl of PBS before flow cytometry analysis. Labeled cells were analyzed on a FACscan apparatus (Becton Dickinson and Co.).

Preparation of DC2.4 exosomes. Exosomes were isolated from the supernatant of the DC2.4 cell line by differential ultracentrifugation by following a procedure described by Raposo et al. (20).

Briefly, DC2.4 cells were washed and recultured in fresh medium with or without TAg for 18 h. The supernatants were collected and centrifuged for 10 min at 300 × *g* to remove the cells. The supernatants were sequentially centrifuged in a JA 30.50 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 30 min at 4,000 × *g*, for 30 min at 10,000 × *g*, and then for 60 min at 100,000 × *g*. The pellets were then resuspended in 1 ml of 1× PBS and centrifuged for 60 min at 100,000 × *g*.

The pellets were resuspended in 200 μl of 1× PBS, and the protein content was determined by using a Micro-BCA kit (Sigma). Approximately 5 μg of exosomes was routinely obtained from the supernatant of a 3-day culture containing 10^6 DC2.4 cells, and there was no difference between the TAg-pulsed and unpulsed DC preparations. The purity of the exosome preparations was confirmed by electron microscopy.

Electron microscopy of exosomes. The exosome preparation was deposited onto carbon-coated copper grids for 1 min before being washed with distilled water. The grids were then negatively stained for 1 min with a solution of 2% uranyl acetate in distilled water. After being dried thoroughly, the grids were observed with a JEOL (Tokyo, Japan) 1010 XC electron microscope.

Homing of exosomes. Fifty micrograms of DC2.4 cell-derived exosomes or 5×10^6 DCs were labeled with 50 μCi of ^{51}Cr for 1 h at 37°C, and then washed to remove any unincorporated ^{51}Cr. Fifty micrograms of DC2.4 cell-derived exosomes or 5×10^6 DCs were injected into the tail veins of the recipient mice. The mice were killed 2, 6, or 24 h later. Blood and urine were collected, and the intestine, lungs, kidneys, liver, spleen, brain, and peripheral (superficial, inguinal, mesenteric, brachial, and popliteal) lymph nodes were removed. The organs were carefully homogenized in 3 ml of water–1% Triton X-100, and the radioactivity in all of the organs was counted with a gamma counter. Values are expressed as percentages of the radioactivity found in the organ relative to that in the rest of the body. Two mice were used in each experiment, and each experiment was performed at least twice.

Exosome immunizations and *Toxoplasma* challenge. In all of the experiments, the mice were given, at an interval of 15 days, two equivalent intravenous doses of 10 μg of TAg-pulsed- or unpulsed-DC2.4 cell-derived exosomes, 2.5×10^5 TAg-pulsed or unpulsed DCs, or 10 μg of TAg prepared by using the same ultracentrifuging procedure as for exosome preparation. Control mice were untreated. All mice were orally infected on day 10 after the second immunization, either with 30 cysts of *T. gondii* 76K or with 10 cysts of *T. gondii* 76K (a sublethal dose).

In order to study cellular immune responses, the spleens were harvested on day 10 after the second immunization, before the mice were infected.

Sera were collected on day 21 after the second immunization in order to study the humoral immune response.

Mice challenged with 30 cysts were observed daily for mortality. One month after the challenge with 10 cysts, the mice were killed and their brains were recovered. Each brain was homogenized in 5 ml of PBS with a mortal and pestle. The cysts in each brain homogenate were counted under the microscope. The results for the different groups are expressed as means ± standard deviations.

Immune serum Western blotting. Serum samples were collected from the retro-orbital sinuses of mice on day 21 after the second immunization and were used for immunoblot analysis. Electrophoresis and immunoblotting of purified *T. gondii* tachyzoites as the source of antigen were performed as previously described (3). *T. gondii* antigens recognized by MAbs were detected by using a goat anti-mouse IgG or an anti-mouse IgM, alkaline phosphatase conjugate (Sigma).

Measurement of the antigen-specific proliferative response. The spleens were harvested on day 10 after the second immunization and pressed through a stainless steel mesh. Single-cell suspensions were obtained by filtering samples through a nylon mesh to remove tissue debris. A hypotonic shock was administered to remove spleen erythrocytes. Splenocytes were suspended in RPMI 1640 (GIBCO, Cergy-Pontoise, France) supplemented with 5% FCS, HEPES (25 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), β-mercaptoethanol (5×10^{-5} M), and penicillin-streptomycin (100 μg/ml), and seeded in triplicate in flat-bottomed 96-well microtitration plates (Costar, Brumath, France) at 5×10^5 cells per well in 200 μl of culture medium, alone or containing various concentrations of TAg. Ten micrograms of concanavalin A/ml was used to confirm proliferation. The plates were incubated for 3 days in 5% CO_2 at 37°C, and 1 μCi of [^{3}H]thymidine/well was added. After a further 18 h, the cells were collected on fiberglass filters, and the radioactivity (counts per minute) was determined by liquid scintillation counting. The proliferation was expressed as the stimulation index (counts per minute for stimulated cells/counts per minute for unstimulated cells).

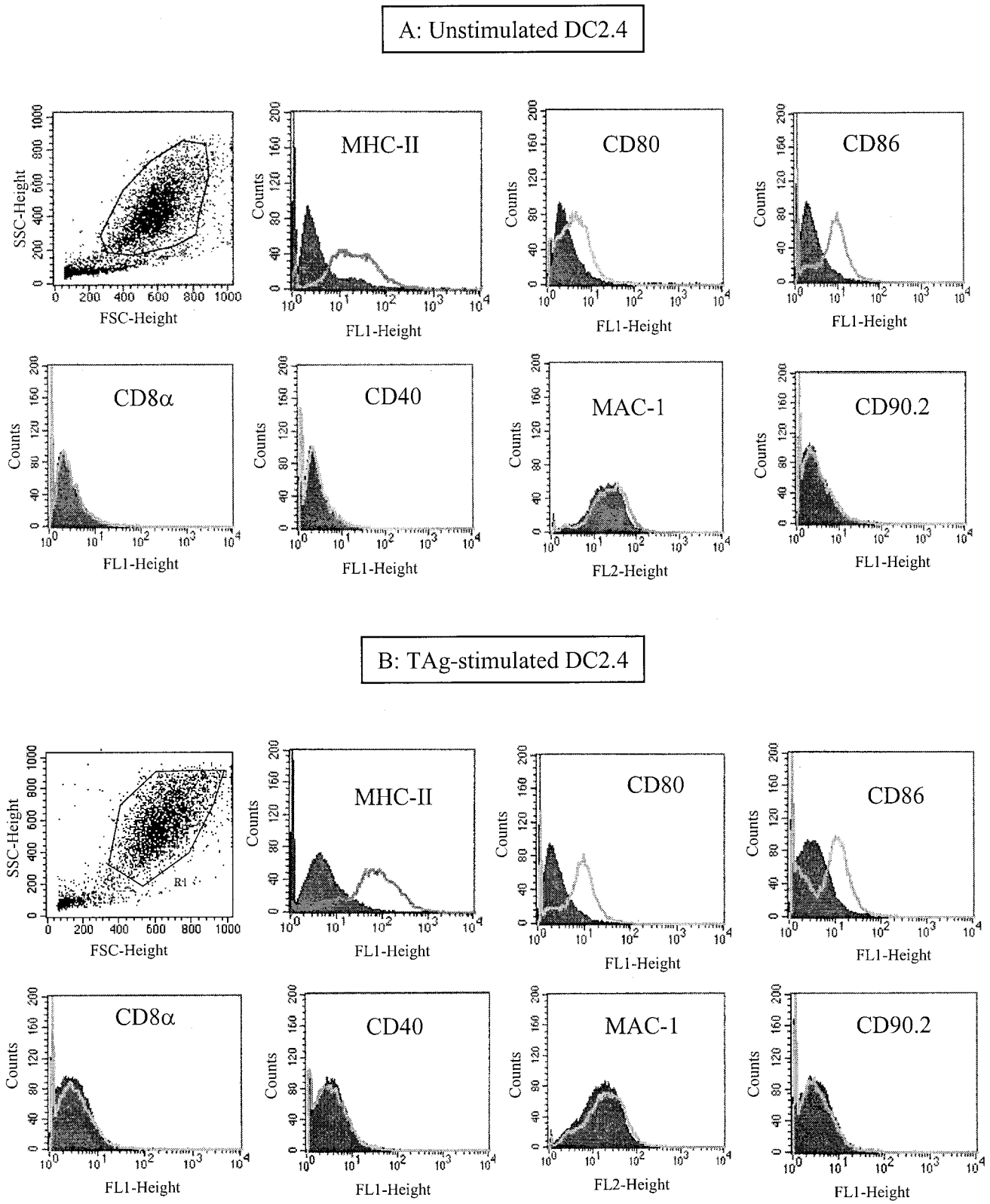
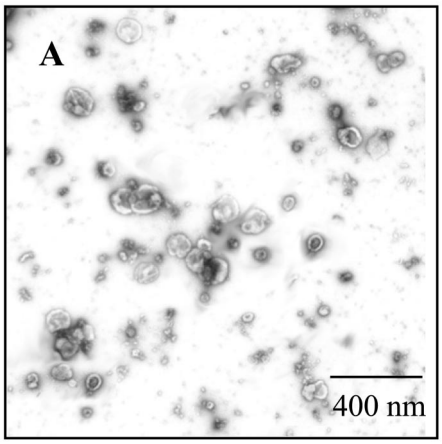


FIG. 1. Immunofluorescence analysis of DC2.4 cell markers. TAg-pulsed DCs express more MHC class II molecules and CD80 than unpulsed DCs. Both express the same level of CD86, and neither expresses CD8α, CD40, MAC-1, or CD90.2. Shaded peaks represent the results of cell staining with the isotype standard only. These data are representative of three experiments with similar results. FSC, forward scatter; SSC, side scatter; FL1 and -2, fluorescence experiments 1 and 2, respectively.



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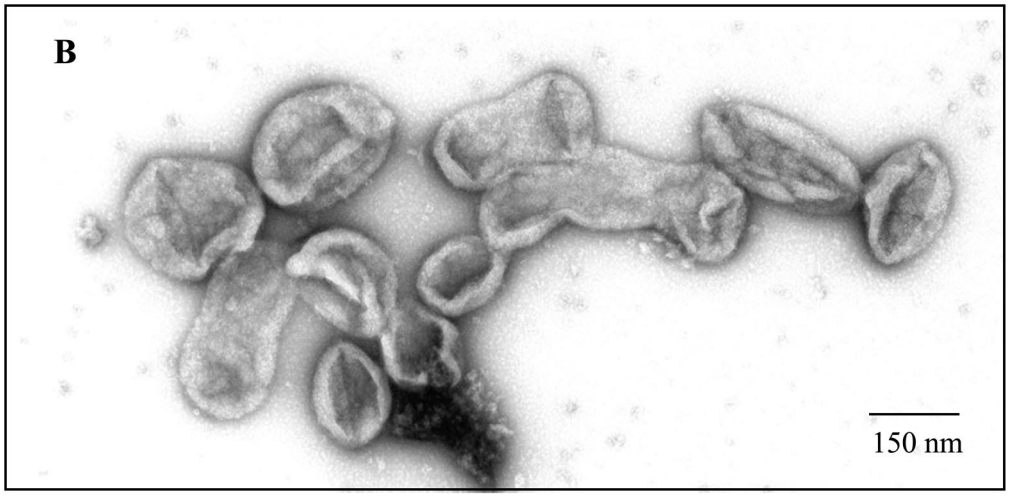

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FIG. 2. Negative staining of DC-derived exosomes obtained by differential ultracentrifugation of a 24-h culture of DC2.4 cell supernatant; 60- to 150-nm-diameter vesicles were observed in an isolated (A) or congregated (B) form.

Determination of the concentration of cytokine. The spleens were cultured in 24-well plates at 5×10^6 cells/well in 1 ml of culture medium alone or containing 10 μg of TAg/ml. The cell-free culture supernatants were harvested and assayed for IL-2 (at 24 h), IL-4 (at 24 h), IL-5 (at 48 h), IL-10 (at 96 h), and IFN-γ (at 72 h) activities. The cytokine concentrations were determined by using a commercial enzyme-linked immunosorbent assay kit (DuoSet; Genzyme, Cergy-Pontoise, France) according to the manufacturer's instructions, with reference to standard curves corresponding to known amounts of mouse recombinant IL-2, IL-4, IL-5, IL-10, and IFN-γ. The sensitivity limits for the assays were 50 pg/ml for IL-10, 20 pg/ml for IFN-γ and IL-5, and 10 pg/ml for IL-4 and IL-2.

Statistical analysis. The statistical significance of the differences between groups was determined by Student-Newman-Keuls multiple comparisons test.

RESULTS

Cell surface markers on TAg-pulsed or unpulsed DC2.4 cells. As already shown (24), the DC2.4 cell line, which lacked T-cell-specific (CD3) and B-cell-specific (surface Ig) markers, expressed high levels of MHC class I and class II molecules and of the costimulatory molecules CD80 and CD86 (Fig. 1A), as well as of CD32 (FcγRII) and CD54 (ICAM-1). These cells are also known to express the specific marker for murine DCs (DEC-205; 33D1) (24). We demonstrated an increase of CD80 and MHC class II molecule expression by DC2.4 cells that had previously undergone TAg stimulation (Fig. 1B), whereas no differences in levels of expression of CD86, CD8α, CD40, MAC-1, and CD90.2 were observed between TAg-pulsed and unpulsed DC2.4 cells.

Exosome purification. After centrifugation of the supernatants of the DC2.4 cell line at different multiples of gravity, small vesicles were observed by electron microscopy. Consistent with the findings reported by Zitvogel et al. (39), a homogeneous population of vesicles 60 to 150 nm in diameter was observed (Fig. 2). About 5 μg of exosomes, depending on the protein content, was routinely obtained from the supernatant of a 24-h culture containing 10^6 TAg-pulsed or unpulsed DC2.4 cells. No significant differences were found in the concentrations of exosomes from pulsed and unpulsed DC2.4 cells.

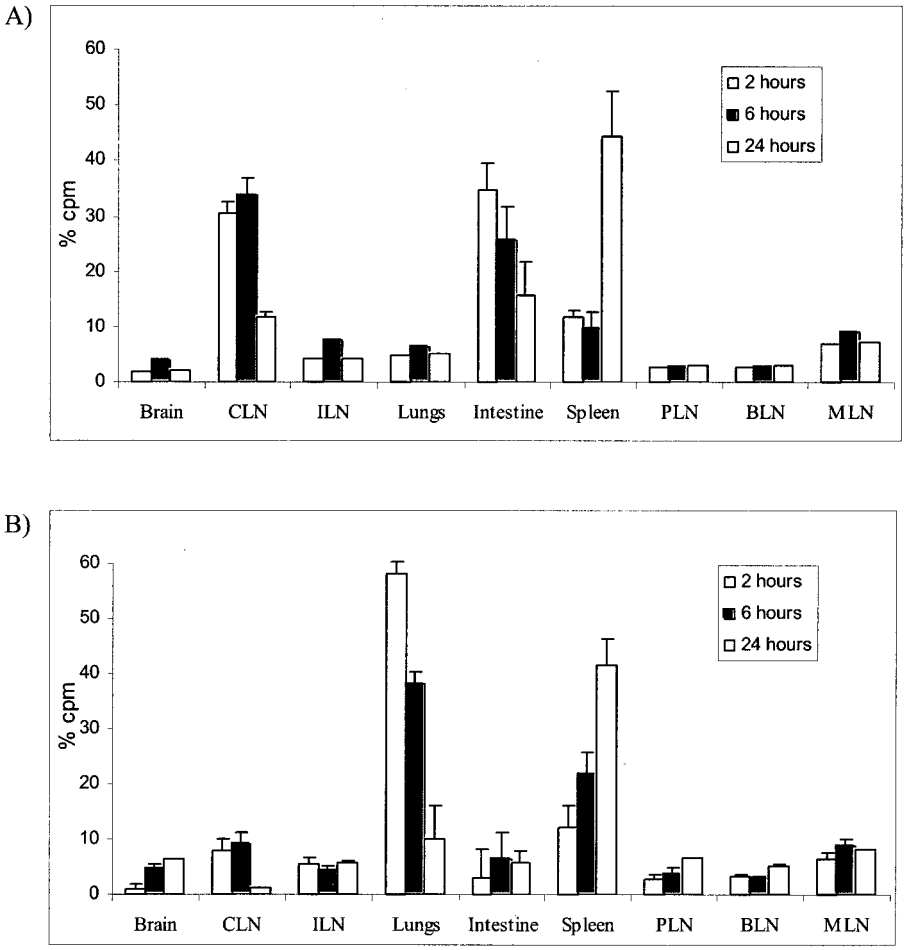

FIG. 3. Fifty micrograms of TAg-pulsed-DC2.4 cell-derived exosomes (A) or 5×10^6 TAg-pulsed-DC2.4 cells (B) were incubated with ^{51}Cr before injection into C57BL/6 mice. At 2 h (first white bar for each sample), 6 h (black bar), or 24 h (second white bar for each sample) after the transfer, radioactivity was measured in each organ. Levels of homing to different organs were compared. Results are expressed as the percentages of radioactivity recovered from a specific organ out of the total radioactivity in the mouse. Results from one of three similar experiments are shown and are expressed as means ± standard errors of the means ($P < 0.05$). CLN, cervical lymph node; ILN, inguinal lymph node; PLN; popliteal lymph node; BLN, brachial lymph node; MLN, mesenteric lymph node.

Homing of DC2.4 cell-derived exosomes following adoptive transfer. To explore the relationship between the triggering of the immune response in the various compartments and the relocalization of exosomes after adoptive transfer, a radioisotope trafficking study was done (Fig. 3A). A similar study was performed with labeled DCs for comparison (Fig. 3B).

Two hours after intravenous injection with ^{51}Cr-labeled-DC2.4 cell-derived exosomes, less than 5% of the total radioactivity was found in the lungs (Fig. 3A). Thirty-five percent of the total radioactivity was detected in the intestines of the recipient mice, 30% was detected in the cervical lymph nodes, 12% was detected in the spleens, and 6% was detected in the mesenteric lymph nodes. The localization of DC exosomes in cervical lymph nodes has yet to be explored. After 24 h, we observed a reduction in the percentage of total radioactivity in the cervical lymph nodes (which decreased to 10%). Moreover, after both 6 and 24 h, we observed reductions in the percentages of total radioactivity in the intestines (to 25 and 16%, respectively), whereas the radioactivity in the spleen had markedly increased after 24 h (to 45%) and remained stable in the mesenteric lymph nodes (at 9 and 8% after 6 and 24 h, respectively). The radioactivity in other compartments was always ≤5% at 2, 6, and 24 h after injection.

Two hours after the intravenous injection of 5×10^6 ^{51}Cr-labeled DC2.4 cells, 58% of the total radioactivity was detected in the lungs of the recipient mice, 12% was detected in the spleens, 8% was detected in the cervical lymph nodes, and 6% was detected in the mesenteric lymph nodes (Fig. 3B). After 6 and 24 h, we observed a diminution of the percentages of the total radioactivity in the lungs (to 38 and 9%, respectively) and cervical lymph nodes (to 1.5% at 24 h), whereas the percentages of the total radioactivity had increased in the spleen (to 22 and 41% after 6 and 24 h, respectively) and was stable in the mesenteric lymph nodes (at 9 and 8% after 6 and 24 h, respectively). The radioactivity in other immune compartments was always ≤5% at 2, 6, and 24 h after injection.

These results demonstrate that the distribution of DC2.4 cells and their exosomes had changed as soon as 2 h after

intravenous adoptive transfer, since the exosomes were detected in both the intestinal and systemic compartments, whereas DCs were found only in the systemic compartments.

TAgs recognized by serum IgM and IgG antibodies of mice immunized with exosomes. Serum anti-*T. gondii* IgM antibodies (Fig. 4A) were detected only in mice injected twice 21 days earlier with TAg-pulsed DC2.4 cells (lane 4) or with TAg-pulsed-DC2.4 cell-derived exosomes (lane 6). The serum IgM antibody response was directed exclusively against antigens with apparent molecular masses of 30 kDa (SAG1). This band was not detected by serum IgM antibodies from mice injected 21 days earlier with unpulsed DC2.4 cells (lane 3) or with unpulsed-DC2.4 cell-derived exosomes (lane 5). Serum anti-*T. gondii* IgM antibodies from mice infected 21 days earlier with *T. gondii* (lane 7) recognized a 30-kDa antigen as previously described (3).

Serum anti-*T. gondii* IgG antibodies (Fig. 4B) were detected only in samples from mice injected twice 21 days earlier with TAg alone (lane 2), with TAg-pulsed DC2.4 cells (lane 4), or with TAg-pulsed-DC2.4 cell-derived exosomes (lane 6). The serum IgG antibody response was directed against antigens with apparent molecular masses of 21, 30, and 38 kDa for mice immunized with TAg-pulsed DC2.4 cells (lane 4) and of 21, 30, 38, and 55 kDa for mice immunized with TAg-pulsed-DC2.4 cell-derived exosomes (lane 6). None of these bands was detected by serum IgG antibodies from mice injected 21 days earlier with unpulsed DC2.4 cells (lane 3) or with unpulsed-DC2.4 cell-derived exosomes (lane 5). Serum anti-*T. gondii* IgG antibodies from mice infected 21 days earlier with *T. gondii* (lane 7) recognized a 30-kDa antigen as previously described. The 21-, 30-, 38-, 55-, and 60-kDa antigens recognized by anti-*T. gondii* serum IgG antibodies showed a migration pattern similar to that of the main TAgs (SAG2, SAG1 [lane 8], SAG3, ROP2, and ROP4, respectively).

It was therefore concluded that the intravenous administration of TAg-pulsed-DC2.4 cell-derived exosomes triggered humorally mediated immunity to *T. gondii*.

Cellular proliferative response in spleen cells from mice immunized with exosomes. TAg-induced cellular proliferation was assayed with spleen cells from mice immunized twice with 10 μg of unpulsed- or TAg-pulsed-DC2.4 cell-derived exosomes 10 days after immunization (Fig. 5A). Proliferation was also assayed with spleen cells from mice immunized twice with 2.5×10^5 unpulsed or TAg-pulsed DC2.4 cells or 10 μg of TAg alone.

A strong proliferative response to TAg restimulation was observed in spleen cells (stimulation index, 22.5) of mice immunized with 10 μg of TAg-pulsed-DC2.4 cell-derived exosomes. Proliferation was also observed (stimulation index, 10.5) in mice treated with TAg-pulsed DC2.4 cells (Fig. 5A). Insignificant proliferation was observed in spleen cells from mice given unpulsed DC2.4 cells (stimulation index, 2) or TAg alone (stimulation index, 1.7) (data not shown), and no proliferation was observed in spleen cells from untreated mice or from mice given unpulsed-DC2.4 cell-derived exosomes.

Cytokine production by spleen cells from mice immunized with exosomes. The supernatants of cultured immune spleen cells from TAg-pulsed- or unpulsed-DC2.4 cell-derived exosomes were evaluated for the production of IL-2, IL-4, IL-5, IL-10, and IFN-γ in response to TAg on day 10 (Fig. 5) after immunization (before infection).

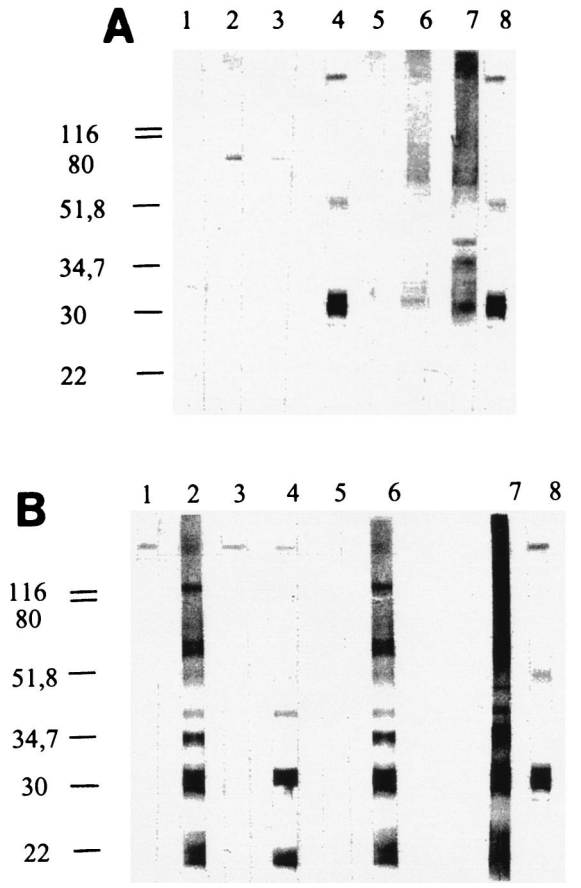

FIG. 4. Western blot analysis of *T. gondii* antigens recognized by serum IgM (A) and serum IgG (B) antibodies. Sera were collected from untreated mice (lane 1); from mice treated with TAg antigen (lane 2), unpulsed DC2.4 cells (lane 3), TAg-pulsed DC2.4 cells (lane 4), unpulsed-DC2.4 cell-derived exosomes (lane 5), and TAg-pulsed-DC2.4 cell-derived exosomes (lane 6); and from mice infected 21 days earlier with *T. gondii* cysts (strain 76K) (lane 7). The MAb 1E5 (anti-P30 [SAG1]) was used as a control (lane 8). The molecular masses (in kilodaltons) of protein standards are given on the left.

Th2 cytokines (IL-10, IL-4, and IL-5). Ten days after immunization (Fig. 5), spleen cells from C57BL/6 mice immunized with TAg-pulsed-DC2.4 cell-derived exosomes produced less IL-10 (<0.05 ng/ml) in response to TAg restimulation (Fig. 5B) ($P < 0.001$) than spleen cells from untreated mice (2.8 ng/ml) or mice given unpulsed-DC2.4 cell-derived exosomes (1.5 ng/ml). Spleen cells from C57BL/6 mice immunized with TAg-pulsed-DC2.4 cell-derived exosomes and from mice immunized with unpulsed-DC2.4 cell-derived exosomes produced less IL-5 (58 and 72 pg/ml, respectively) in response to TAg restimulation (Fig. 5C) than spleen cells from untreated mice (115 pg/ml) ($P < 0.001$). No specific release of IL-4 was detected (data not shown).

By way of comparison, this experiment was also performed on supernatants from cultured immune splenocytes from TAg-pulsed- or unpulsed-DC2.4 cell-injected mice. Spleen cells from mice immunized with TAg-pulsed DC2.4 cells produced less IL-10 (0.25 ng/ml) in response to TAg restimulation (Fig.

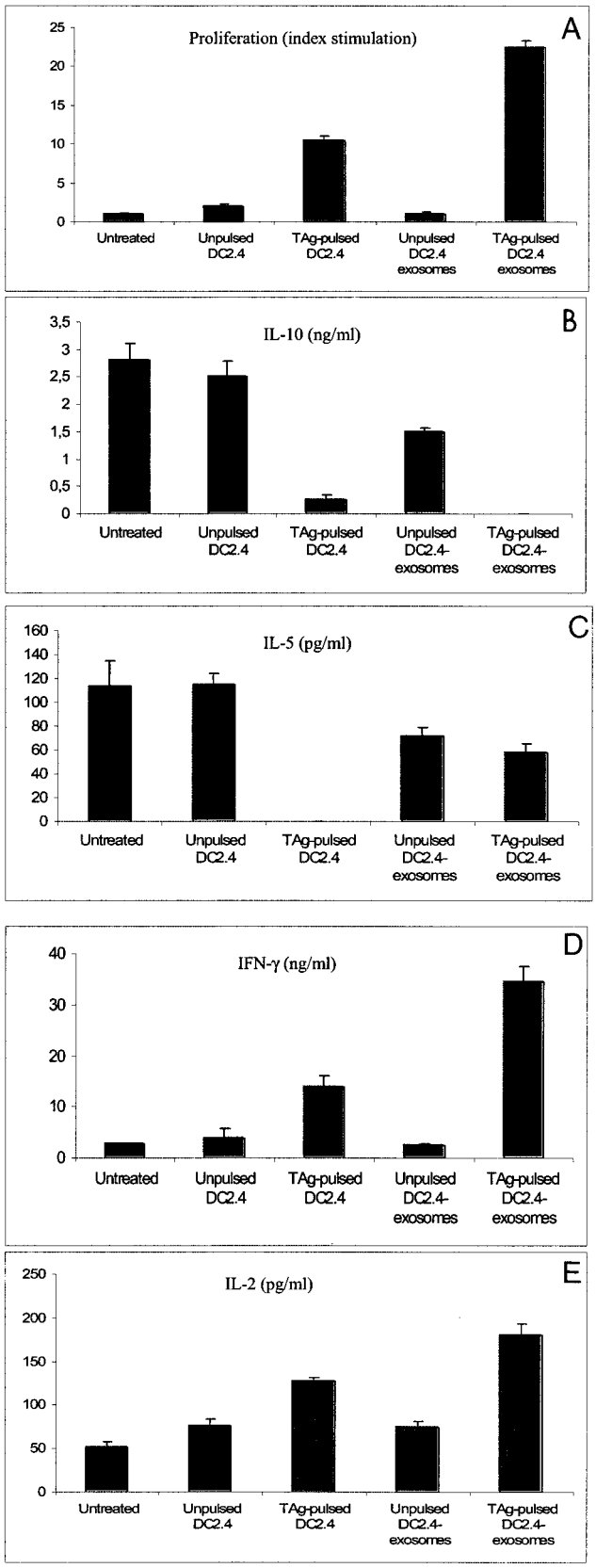

FIG. 5. Cellular proliferative response and cytokine production following intravenous immunization with 10 μg of TAg-pulsed- or unpulsed-DC2.4 cell-derived exosomes or with 2.5×10^5 TAg-pulsed or unpulsed DC2.4 cells, before challenge with 30 cysts of *T. gondii* 76K. At 10 days after immunization, the spleen cells were isolated and stimulated in vitro with TAg (10 μg/ml). Proliferation was assessed after 4 days. Values were measured at 24 h of culture for IL-2, at 48 h for IL-5, at 72 h for IFN-γ, and at 96 h for IL-10. Results are the mean cytokine concentrations or counts per minute for proliferation assays with spleen and mesenteric lymph node cells from three mice per experimental group. Results from one of three similar experiments are shown and are expressed as means ± standard errors of the means ($P < 0.001$).

5B) ($P < 0.001$) than spleen cells from untreated mice (2.8 ng/ml) or mice given unpulsed DC2.4 cells (2.5 ng/ml). Moreover, spleen cells from mice immunized with TAg-pulsed DC2.4 cells produced less IL-5 (<20 pg/ml) in response to TAg restimulation (Fig. 5C) ($P < 0.001$) than spleen cells from untreated mice (115 pg/ml) or mice given unpulsed DC2.4 cells (115 pg/ml). No specific release of IL-4 was detected (data not shown). These findings (reduced production of IL-10 and IL-5 associated with the absence of IL-4 secretion) demonstrate that the intravenous administration of TAg-pulsed DC-derived exosomes and TAg-pulsed DC2.4 cells triggered a reduction in the secretion of Th2 cytokines by the spleen.

Th1 cytokines (IFN-γ and IL-2). The production of IFN-γ (Fig. 5D) by spleen cells from C57BL/6 mice immunized with TAg-pulsed-DC2.4 cell-derived exosomes (34 ng/ml) was greater ($P < 0.001$) than that of untreated mice (2.8 ng/ml) and of mice given unpulsed-DC2.4 cell-derived exosomes (2.5 ng/ml). Finally, the production of IL-2 (Fig. 5E) by spleen cells from C57BL/6 mice given TAg-pulsed exosomes (180 pg/ml) was greater ($P < 0.001$) than that of untreated mice (52 pg/ml) and of mice given unpulsed-DC2.4 cell-derived exosomes (75 pg/ml).

The production of IFN-γ (Fig. 5C) by spleen cells from C57BL/6 mice immunized with TAg-pulsed DC2.4 cells (14 ng/ml) was greater ($P < 0.001$) than that of untreated mice (2.8 ng/ml) and of mice given unpulsed DC2.4 cells (3.7 ng/ml). The production of IL-2 (Fig. 5E) by spleen cells from C57BL/6 mice given TAg-pulsed DC2.4 cells (127 pg/ml) was greater ($P < 0.01$) than that of untreated mice (52 pg/ml) and of mice given unpulsed DC2.4 cells (75 pg/ml).

These findings (increased production of IFN-γ and IL-2) demonstrate that the intravenous administration of TAg-pulsed DC2.4 cells or TAg-pulsed-DC2.4 cell-derived exosomes triggered an increase in the secretion of Th1 cytokines by the spleen. This increase is correlated with a decline in the secretion of Th2 cytokines.

Protection of mice immunized with exosomes against acute infection. The ability of TAg-pulsed-DC2.4 cell-derived exosomes to provide protection against the acute stage of *T. gondii* infection was evaluated with C57BL/6 mice given an oral challenge with 30 cysts of the 76K strain of *T. gondii*. The mice were observed daily for mortality (Fig. 6).

The TAg-pulsed-DC2.4 cell-derived exosomes protected mice against acute infection, with 67% of the mice surviving after an oral challenge with a lethal dose of *T. gondii* cysts (Fig. 6A). In comparison, survival rates were identical (20%) for mice given TAg alone, mice given unpulsed-DC2.4 cell-derived exosomes, and untreated mice.

These observations showed that TAg-pulsed-DC2.4 cell-de-

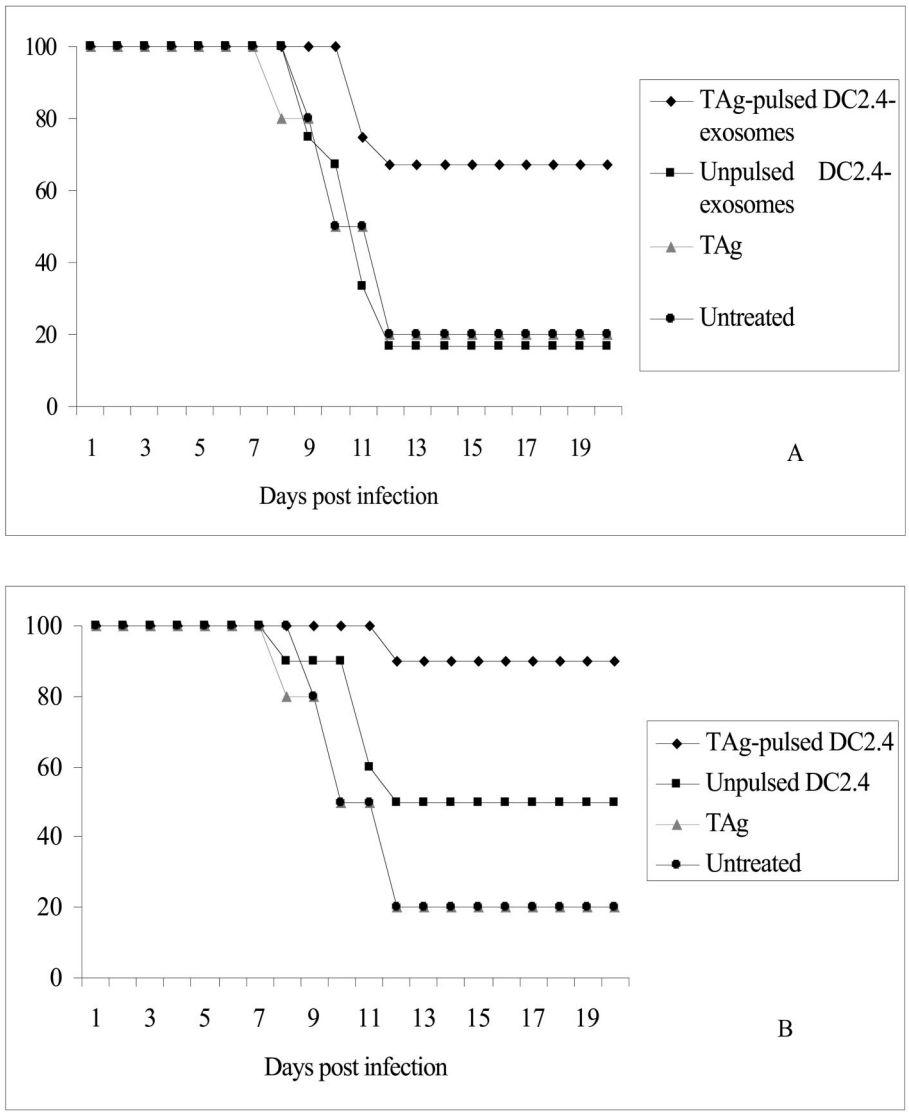

FIG. 6. Survival of C57BL/6 mice following immunization with TAg, unpulsed-DC2.4 cell-derived exosomes, and TAg-pulsed-DC2.4 cell-derived exosomes (A), and with TAg, unpulsed DC2.4 cells, and TAg-pulsed DC2.4 cells (B). Mice immunized twice with 10 μg of TAg, with 2.5×10^5 TAg-pulsed or unpulsed DC2.4 cells, or with 10 μg of TAg-pulsed- or unpulsed-DC2.4 cell-derived exosomes were orally infected with 30 cysts of *T. gondii* 10 days later. Mice were observed daily for mortality. These data are representative of three experiments with similar results.

rived exosomes protected C57BL/6 mice against an oral challenge with *T. gondii*.

We have also demonstrated that TAg-pulsed DCs protected the mice (90%) against acute infection. In comparison, unpulsed DC2.4 cells produced lower survival after a virulent challenge, since 50% of mice died within 12 days postinfection. A lower survival rate (20%) was observed for untreated mice.

Protection of mice immunized with exosomes against chronic infection. The long-term protection conferred by pulsed-DC2.4 cell-derived exosomes to C57BL/6 mice was evaluated by counting brain cysts 1 month after an oral challenge with 10 cysts (Fig. 7). Untreated infected mice developed a substantial number of intracerebral cysts containing bradyzoites (4,500 ± 800 cysts/brain) (Fig. 7). Mice immunized twice with 10 μg of TAg-pulsed-DC2.4 cell-derived exosomes controlled cyst formations (75% protection), showing only 1,125 ± 200 cysts/brain ($P < 0.001$). TAg-pulsed DCs protected the mice equally ($P > 0.05$) against the chronic stage of infection (765 ± 100 cysts/brain; 86% protection) ($P < 0.001$), whereas TAg alone, unpulsed DCs, and unpulsed-DC2.4 cell-derived exosomes only weakly protected the mice, as shown by the observed 3,500 ± 380 cysts/brain ($P < 0.01$), 3,700 ± 650 cysts/brain ($P < 0.05$), and 3,500 ± 500 cysts/brain ($P < 0.01$), respectively (Fig. 7).

We can therefore conclude that exosomes derived from DC2.4 cells loaded with TAg were effective in inducing considerable resistance to cyst formation.

DISCUSSION

We studied the ability of DC-derived exosomes to confer protective immunity against toxoplasmosis. We showed that

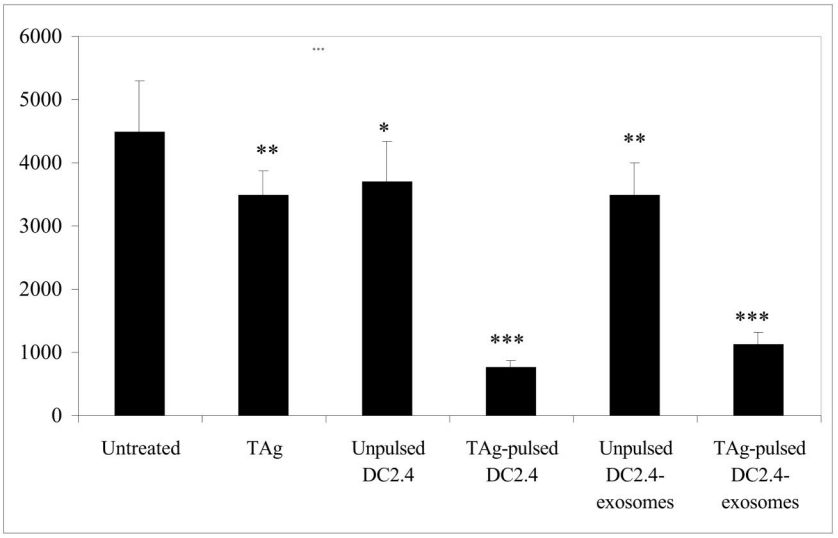

FIG. 7. Assay for protection against chronic toxoplasmosis (oral challenge). C57BL/6 mice were immunized twice with 2.5×10^5 Tag-pulsed or unpulsed DC2.4 cells, with 10 μg of TAg-pulsed- or unpulsed-DC-derived exosomes, or with 10 μg of TAg only. C57BL/6 mice were orally infected with 10 cysts of *T. gondii* 10 days later. The cyst burden was determined by counting brain cysts at 30 days postchallenge; standard deviations are noted. These data are representative of three experiments with similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the injection of exosomes derived from DC2.4 cells which had been pulsed ex vivo with *T. gondii* antigens induced a systemic *Toxoplasma*-specific Th1-modulated immune response in vivo and conferred protection against *Toxoplasma* infection. We have shown that these exosomes are transferred preferentially and rapidly through the intestine and cervical lymph nodes before reaching the spleen compartment. This study is the first to report an anti-infectious effect of DC-derived exosomes.

We demonstrated that TAg-pulsed-DC2.4 cell-derived exosomes induced good protection against the acute stage of *T. gondii* infection, since 67% of C57BL/6 mice that had been treated with these exosomes survived compared to 18% of those treated with unpulsed-DC2.4 cell-derived exosomes. Moreover, we showed that these C57BL/6 mice treated with exosomes derived from antigen-pulsed DC2.4 cells and challenged with a sublethal dose of *T. gondii* cysts exhibited a dramatically lower cerebral parasite burden (75%) following parasite infection than that of untreated mice or mice given exosomes derived from unpulsed DC2.4 cells, although the degree of pathology in the brains remains to be addressed. As TAg-pulsed DCs have already been shown to provide protection against a *T. gondii* challenge (4), we also investigated the induction of a protective response after the adoptive transfer of antigen-pulsed DC2.4 cells that can be used as a positive control. Adoptively transferred DC2.4 cells pulsed ex vivo with TAg induce greater protection against both the acute (90% protection) and chronic (86%) stages of the infection than unpulsed DC2.4 cells (50 and 18% protection, respectively). The partial protection induced by unpulsed DC2.4 cells against the acute stage of *Toxoplasma* infection (50%) has yet to be investigated and may indicate the existence of innate nonspecific immunity.

The protection obtained with TAg-pulsed-DC2.4 cell-derived exosomes was closely related to the development of a systemic Th1 immune response. These exosomes were able to induce *T. gondii* antigen-specific proliferation in the spleen compartment, which produced IFN-γ and IL-2 and down-regulated IL-5 and IL-10. The proliferative response was observed as early as day 10 after the second immunization and persisted throughout the infection (data not shown).

The good protection observed after TAg-pulsed-DC2.4 cell adoptive transfer was also correlated to the production of a Th1-cytokine profile via the production of IL-2 and IFN-γ and the down-regulation of IL-10 and IL-5 in spleen compartments. These data concerning DC immunization (2.5×10^5 DCs/mouse) must be considered a control experiment rather than a quantitative comparison with exosome immunization (10 μg/mouse).

Our results showing a systemic Th1 immune response are therefore in agreement with those reported by many different groups (8), demonstrating that the protective systemic immunity to *T. gondii* infection is cell mediated, with the particular specific involvement of IFN-γ. This protective capacity may be associated with DCs or exosome trafficking. The transport of antigens by APCs from the periphery into organized lymphoid tissues is crucial for triggering an immune response. DCs are probably the main APCs contributing to this antigen transport, as they migrate and home to the T-cell areas of lymphoid tissues after taking up antigens in the periphery (11). We carried out a radioisotope trafficking study to explore the relationship between the triggering of an immune response in the various compartments and the relocation of DC2.4 cells or exosomes after adoptive transfer and to monitor the migration pathway of these cells or exosomes through various organs. Our findings showed that DC2.4 cell-derived exosomes were located in the intestines, the spleens, and, surprisingly, the cervical lymph nodes of recipient mice at an early stage. After 6 and 24 h, the number of DC2.4 cell-derived exosomes in the cervical lymph nodes and intestine seemed to fall as the exosomes reached the spleen, where the radioactivity had increased considerably. The presence of exosomes in the spleen may be a final prerequisite for triggering the immunological

response observed in the systemic compartment, which leads to protection. Immediately after DC immunization, 58% of DC2.4 cells were detected in the lungs of the recipient mice and 12% were detected in their spleens, whereas less than 5% radioactivity was detected in their lungs 2 h after intravenous injection of DC2.4 cell-derived exosomes. Kupiec-Weglinski et al. (10) also observed that intravenously injected purified spleen DCs were immediately sequestered in the lung but then actively migrated into the liver and into the spleen, which is the principal site of DCs. The percentage of DC2.4 cells in the lung had fallen after 6 and 24 h, whereas it had risen markedly in the spleen. This concentration in the spleen may account for the strong immune response that developed in the spleen after immunization.

The mechanisms by which DC2.4 cells or DC2.4 cell-derived exosomes home to appropriate organs are not known. Thery et al. have established the first extensive protein map of DC-derived exosomes. One category of exosome-associated proteins is that involving targeting, docking, and/or fusion with other cells. These cell-specific transmembrane proteins include ICAM-1 and -2, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ for MFG-E8/lactadherin, and an EGF-like growth factor receptor for CD9 (34) and are probably involved in exosome homing. No study has yet investigated exosome migration, and we do not know why the DC-derived exosomes migrate towards the intestine, nor if they are picked up by other cells from the intestine. The ability of exosomes to cross 3-μm-pore-size filters suggests that exosomes can migrate through the intestinal basement membrane to convey antigenic information to the mucosal and systemic immune cells (35). Using a systemic proteomic approach, Thery et al. (34) identified 21 exosomal proteins and were able to propose several possible mechanisms. Exosomes may directly stimulate T cells (7) or be captured by other APCs (32) to amplify the dispersal of peptide-loaded MHC molecules. This capture of exosomes may be governed by the expression at the exosome surfaces of proteins that are known to bind to ligands on other membranes: ICAM-1 and -2 for MAC-1 (1), integrins $\alpha_v\beta3$ and $\alpha_v\beta5$ for MFG-E8/lactadherin (9, 31), and an EGF-like growth factor receptor for CD9 (18, 37). These proteins could be involved in exosome targeting and/or fusion with other cells.

Our findings suggest that the presence of exosomes at the site of antigen exposure is crucial if an effective systemic response is to develop. In order to devise an ideal vaccination protocol for inducing protective immune responses, the route of administration needs to be addressed, since it could affect the homing properties of DCs. Exosomes could therefore provide a new method for communication and for the exchange of antigenic information between cells in the immune system. Indeed, Wolfers et al. (37) demonstrated that exosomes concentrate a set of shared tumor rejection antigens which are efficiently taken up and cross-presented by MHC class I molecules on DCs. Moreover, MFG-E8/lactadherin, the major exosomal component, binds integrins expressed by DCs and macrophages, suggesting that it may be involved in the fusion of exosomes to the membrane of APCs.

In conclusion, these results support the idea that DC-derived exosomes could be used for *T. gondii* immunoprophylaxis and for immunoprophylaxis against many other pathogens.

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